Acta Poloniae Pharmaceutica – Drug Research, Vol. 68 No. 6 pp. 853–858, 2011 ISSN 0001-6837
Polish Pharmaceutical Society

SPECTROPHOTOMETRIC DETERMINATION OF BACITRACIN IN BULK DRUG AS DABSYL DERIVATIVE IN A RANGE OF VISIBLE LIGHT

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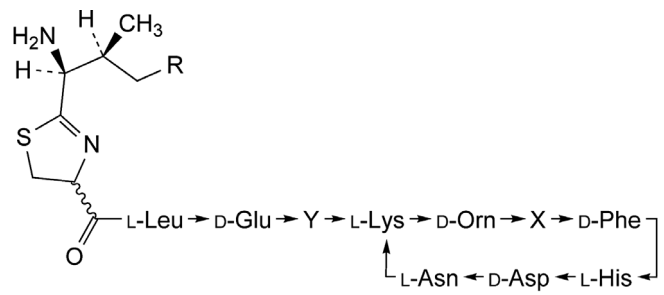
Abstract: A fast spectrophotometric method has been developed for bacitracin identification and determination after condensation reaction with dabsyl chloride. In addition, determination of dye stability of sulfonamide derivative and identification of the molar ratio of reagents was done at various time-points. The developed method has a good linearity with very broad spectrum, correlation coefficient of $r = 0.9972$, good precision (RSD = 1.54 ± 0.11%), and recovery at three different levels of concentration was found between 98.33% and 103.47%. Usefulness of the method was demonstrated by positive results obtained during determination of bacitracin concentration in bulk drug.

Keywords: bacitracin, dabsyl chloride, VIS spectrophotometry.

Bacitracin from *B. licheniformis* and *B. subtilis* strains represents a mixture of nine cyclic polypeptides and the most important component is bacitracin A. It has bactericidal properties against Gram (+) bacteria and is used in treatment of skin, eye, and ear infections. Because bacitracin demonstrates local action and is not absorbed through mucous membrane, then it is possible to use it in treatment of patients with pseudomembranous enterocolitis. Bacitracin is mostly administered in the form of drops and ointments, in complex preparations with neomycin (1, 2). Structure of bacitracin A used in therapy is presented in Figure 1.

Since 1975, dabsyl chloride (DBS) is used for identification of N-terminal amino acid in polypeptide chain during analysis of compounds containing amine group (3). The first step in the process is condensation of DBS with polypeptide through N-terminal amino acid. The second step is hydrolysis in acidic environment that results in a product – sulfon-



Name	Molecular formula	X	Y	R
Bacitracin A	$C_{66}H_{103}N_{17}O_{16}S$	L-Ile	L-Ile	CH_3
Bacitracin B1	$C_{65}H_{101}N_{17}O_{16}S$	L-Ile	L-Ile	H
Bacitracin B2	$C_{65}H_{101}N_{17}O_{16}S$	L-Val	L-Ile	CH_3
Bacitracin B3	$C_{65}H_{101}N_{17}O_{16}S$	L-Ile	L-Val	CH_3

Figure 1. Structure and different types of bacitracin according to FP VIII

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amide derivative. The final, third, step is chromatographic identification of obtained product. This reaction is highly sensitive (10^{-9} – 10^{-10} mol/dm^3).

Published data demonstrate wide application of chromatographic techniques with varied methods of detection like HPLC-MS, HPLC-UV, MEKC (4–10). TLC method with densitometric detection with co-occurrence of neomycin in the study material was also used in the quantitative determination of bacitracin (11, 12).

In this paper, the quantitative determination of bacitracin after condensation reaction with dabsyl chloride is presented. Modification in the method of N-terminal amino acid determination with the use of DBS was done by exclusion of hydrolysis and establishing reaction conditions to enable direct spectrophotometric estimation of the product of DBS reaction with the antibiotic.

ANALYSIS

Reagents

Bacitracin from *B. licheniformis* (Sigma, St. Louis, USA), dabsyl chloride = 97.5% (AT) (Fluka

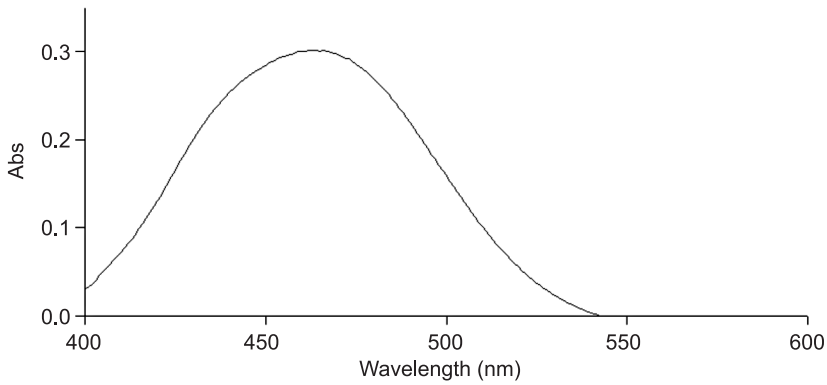

Figure 2. The UV spectrum of the product of bacitracin-DBS condensation.

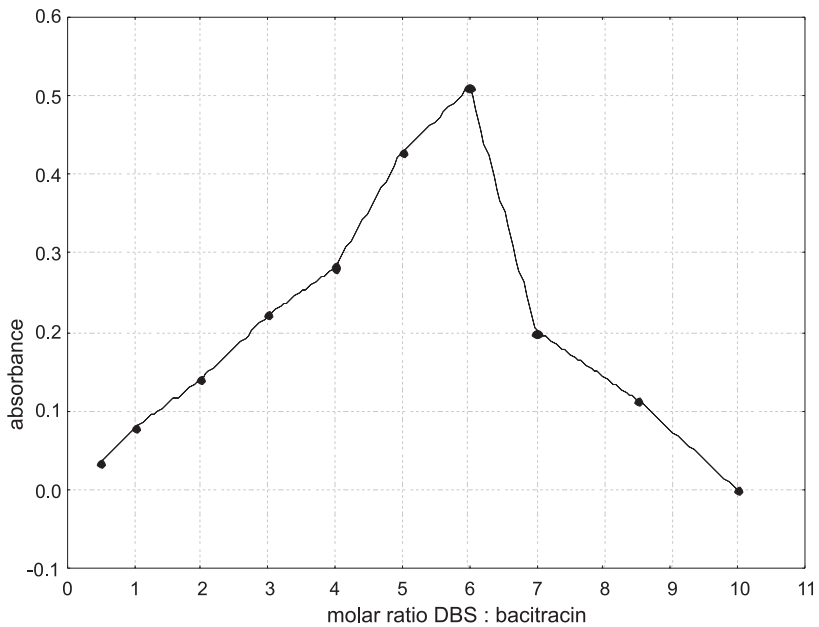

Figure 3. Changes in the absorbance dependent on the molar ratio of DBS : bacitracin

Chemie AG, Buchs, Switzerland), acetone p.a. sodium carbonate p.a. and sodium bicarbonate p.a. (POCh Gliwice, Poland).

Bacitracin used for the experiments met the requirements described in a monograph in Polish Pharmacopoeia (FP VIII) in the scope of identity, purity and biological activity.

Instruments and software

UV/Vis spectrophotometer Cary 100 Conc. Varian, 10 mm glass cells, STATISTICA 9 software.

Formation of bacitracin derivative with dabsyl chloride

A product of reaction was formed when 0.06 mL of bacitracin solution (1×10^{-3} mol/dm^3) in carbonate buffer (pH = 9.0) was mixed with 0.6 mL of dabsyl chloride solution (1×10^{-3} mol/dm^3), heated up at 70^{o}C for 15 min, then cooled down to 25^{o}C and adjusted with acetone to the final volume of 5.0 cm^3. The product demonstrated characteristic absorption spectrum in a range 400 nm to 800 nm with maximum at λ = 474 nm.

Absorption spectrum of the formed product, recorded in the presence of reference material prepared in the same way as a study sample, is presented in Figure 2.

In the above-described conditions, no absorption of separate bacitracin and DBS solutions was recorded in a studied range of measurement. Therefore, it may be assumed that the absorbance value at the maximum of absorption originates from a product obtained in a reaction of the studied reagents.

Further studies estimated the product in the context of its use to develop a new method.

Determination of the molar ratio of reagents

In flasks of 5 cm^3 volume, a series of 9 solutions with the same concentration of bacitracin (2×10^{-5} mol/dm^3) and increasing concentration of DBS ($1\times10^{-5} - 2\times10^{-4}$ mol/dm^3) was prepared. A reference material containing identical volume of carbonate buffer (pH = 9.00) and DBS concentration as in the study sample was prepared for each individual sample. The samples and their references were heated up in water bath at 70^{o}C for 15 min, and then cooled down and adjusted with acetone to the final volume of 5 cm^3. Absorbance was measured in relation to appropriate reference material. A graph presenting dependence of absorbance at 474 nm towards the molar ratio of DBS to bacitracin was created. This graph indicates quantitative run of the reaction with molar ratio 1:6, bacitracin : DBS (Fig. 3).

Stability of color in solutions

Stability of color in solutions was studied by measurement of samples absorbance at λ = 474 nm

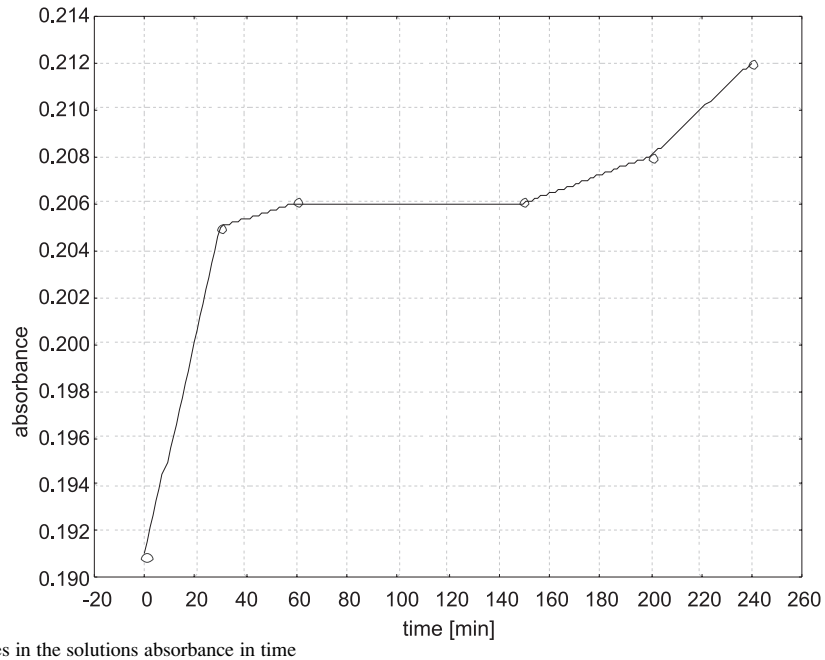

Figure 4. Changes in the solutions absorbance in time

from 0 to 240 min after termination of incubation. The recorded changes of absorbance value at a particular time are presented in Figure 4. Results of these studies confirmed stability of color in solutions after 30 min from termination of incubation which was kept at a constant level for the next 2 h.

Linearity

In order to determine a dependence of absorbance of solutions on the content of antibiotics, a series of solutions with increasing concentration of bacitracin (2×10^{-6} to 2×10^{-5} mol/dm^3) and constant concentration of DBS (1.2×10^{-4} mol/dm^3) were prepared based on the earlier determined molar ratio of reagents. A series consisting of 6 samples containing bacitracin with reference material was incubated in water bath at 70°C for 15 min. Then, the samples were cooled down to 25°C and completed with acetone to 5 cm^3. The absorbance of samples was recorded at λ = 474 nm. A graph representing the dependence of absorbance on concentration of bacitracin in the sample had a good correlation coefficient, r = 0.9972 (Fig. 5).

Absorption coefficient

In the above-described conditions, values of molar absorption coefficient ($\varepsilon_{474nm} = 2.4\times10^4$) and coefficient $A^{1\%}_{1cm} = 168$ were obtained during studies of linearity A = f(c).

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Based on parameters of the curve, the LOD and LOQ (mol/dm^3) values were 1.08×10^{-10} and 3.28×10^{-10},

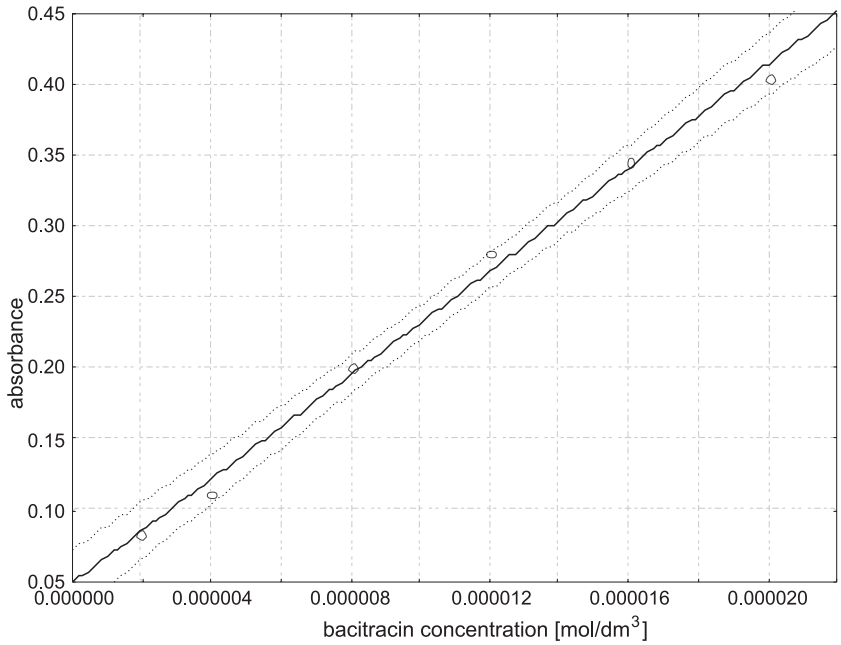

Figure 5. Dependence of the solutions absorbance on bacitracin concentration

Table 1. Precision of the method.

Concentration level of bacitracin in sample	Measured absorbance values	S	RSD [%]
50%	0.1637; 0.1588; 0.1602; 0.1629; 0.1594; 0.1580	2.30×10^{-3}	1.43
100%	0.2875; 0.2849; 0.2861; 0.2795; 0.2921; 0.2906	4.48×10^{-3}	1.56
150%	0.3681; 0.3842; 0.3796; 0.3823; 0.3779; 0.3718	5.81×10^{-3}	1.65

S = standard deviation, RSD = relative standard deviation

Table 2. Bacitracin recovery values expressed as a percentage for three various levels of concentration.

Concentration level of bacitracin in sample	$A_{474\ nm}$	Weighted bacitracin concentration [mol/dm^3]	Measured bacitracin concentration [mol/dm^3]	Recovery [%]	Average recovery [%]
80%	0.2274 0.2279 0.2215	9.60×10^{-6}	9.78×10^{-6} 9.81×10^{-6} 9.46×10^{-6}	101.88 102.17 98.52	100.86
100%	0.2677 0.2648 0.2653	1.20×10^{-5}	1.20×10^{-5} 1.18×10^{-5} 1.19×10^{-5}	100.00 98.33 99.17	99.17
120%	0.3215 0.3090 0.3198	1.44×10^{-5}	1.49×10^{-5} 1.42×10^{-5} 1.48×10^{-5}	103.47 98.61 102.78	101.62

$A_{474\ nm}$ = absorbance at 474 nm

Table 3. The amount of bacitracin in bulk drug.

Average absorbance λ = 474 nm	Bacitracin concentration [mol/dm^3]	Amounts [%]
0.2729	1.22×10^{-5}	101.67
0.2716	1.21×10^{-5}	100.83
0.2648	1.18×10^{-5}	98.33
0.2677	1.19×10^{-5}	99.17
0.2718	1.22×10^{-5}	101.67
0.2653	1.18×10^{-5} 9	8.33
	$x_{sr}= 1.20\times10^{-5}$ $S= 1.90\times10^{-7}$ RSD = 1.58% $\mu_{0.05} = 1.20\times10^{-5} \pm 1.18\times10^{-7}$	$x_{sr}= 100.00$ S= 1.58 RSD= 1.58% $\mu_{0.05} = 100.00 \pm 0.99$

x_{sr} = average [mol/dm^3], S = standard deviation, RSD = relative standard deviation, μ = confidence interval

respectively. These values corresponded to low concentrations of bacitracin and at the same time indicated satisfactory sensitivity of the method.

Precision

For precision determination, six samples with three different concentrations of bacitracin (50%, 100% and 150%) were prepared. The samples with reference material were heated up in water bath at 70^{o}C for 15 min and then cooled down and adjusted with acetone to the scale mark. The absorbance was recorded at λ = 474 nm. The obtained values of absorbance as well as calculated standard deviation (S) and relative standard deviation (RDS) values are presented in Table 1.

Recovery

Recovery was determined based on identified concentration in relation to weighted amount under conditions of developed method. For studies, 3 replicates at three different concentrations of the antibiotic (80%, 100% and 120%) were prepared. The obtained values of absorbance, which were used for estimation of bacitracin recovery, are presented in Table 2.

Determination of bacitracin in bulk drug

A solution of bacitracin (1×10^{-3} mol/dm^3) in carbonate buffer at pH = 9.00 as well as 6 identical mixtures of DBS with bacitracin solutions (1.2×10^{-5} mol/dm^3) were prepared. The absorbance of solutions was recorded at λ_{max} = 474 nm. Concentration of bacitracin in the samples was calculated based on the slope obtained for calibration curve. The obtained values of absorbance and corresponding with them concentrations of bacitracin as well as the percentage ratio to weighted amounts are presented in Table 3.

In bulk drug, concentration of bacitracin was in a range 100 ± 1.67%.

RESULTS AND DISCUSSION

Reaction of free amine groups with dabsyl chloride was used for development of a new method for determination of bacitracin in bulk drug. It was observed that molar ratio of DBS to bacitracin in reaction of condensation was 6:1. The product was stable for 2 h.

The method demonstrated high sensitivity (LOD = 1.08×10^{-10} mol/dm^3 and LOQ = 3.28×10^{-10} mol/dm^3), good precision for various levels of concentration (RSD = 1.43% to 1.65%) and high accuracy expressed as a percentage of recovery (98.33% to 103.47%). The dependence of absorbance on concentration was straight in a range 2×10^{-6} to 2×10^{-5} mol/dm^3. The results of determination of bacitracin in bulk drug are characterized by narrow confidence interval, which is a confirmation of practical usability of the method developed for controlling raw material during production.

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Received: 24. 02. 2011